

**Pro-inflammatory potential of particles from residential
wood smoke and traffic:
Importance of physicochemical characteristics**

by

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Abbreviations

BET	Brunauer, Emmet and Teller
COPD	Chronic obstructive pulmonary disease
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
EELS	Electron energy loss spectroscopy
GM-CSF	Granulocyte macrophage-colony stimulating factor
HR-TEM	High resolution transmission electron microscopy
IL	Interleukin
IL-1R	IL-1 receptor
LAL	Limulus ameobocyte lysate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MIP-2	Macrophage-inflammatory protein 2
NEXAFS	Near-edge X-ray absorption fine structure spectroscopy
SEM	Scanning electron microscopy
SRM	Standard reference material
SAED	Selected area electron diffraction
TEM	Transmission electron microscopy
TLR	Toll like receptor
TNF- α	Tumour necrosis factor α
TNF-R	Tumour necrosis factor receptor
TSP	Total suspended particulate matter
PAH	Polycyclic aromatic hydrocarbon
PM	Particulate matter
RAIAP	Respiratory Allergy and Inflammation due to Ambient Particles
WHO	World Health Organisation
XRMA	X-ray microanalysis

1 List of papers

The thesis is based on the experimental work presented in the papers listed below. The papers will be referred to by their Roman numerals:

- Paper I** Analytical electron microscopy of combustion particles: a comparison of vehicle exhaust and residential wood smoke.
A Kocbach, BV Johansen, PE Schwarze and E Namork.
The Science of the Total Environment, 2005, 346: 231-243.
- Paper II** Physicochemical characterisation of combustion particles from vehicle exhaust and residential wood smoke.
A Kocbach, YJ Li, KE Yttri, FR Cassee, PE Schwarze, E Namork.
Particle and Fibre Toxicology, 2006, 3: 1.
- Paper III** Pro-inflammatory potential of wood smoke and traffic-derived particles in a monocytic cell line.
A Kocbach, E Namork, PE Schwarze.
Toxicology, 2008, 247: 123-132.
- Paper IV** Particles from wood smoke and traffic induce differential pro-inflammatory response patterns in co-cultures.
A Kocbach, JI Herseth, M Låg, M Refsnes, PE Schwarze.
Toxicology and Applied Pharmacology, 2008, 232: 317-326.

Introduction

Over the last decades, the health effects related to exposure to air pollution and particulate matter (PM) have been subject to intensive research. Epidemiological studies have associated exposure to particles with diameters smaller than 10 μm with increased pulmonary and cardiovascular morbidity and mortality (Franklin et al., 2007; Katsouyanni et al., 2001; Metzger et al., 2004; Ostro et al., 2006; Pope III et al., 2002; Zanobetti et al., 2000), and the annual number of premature deaths due to particle exposure has been estimated to be 800.000 worldwide (WHO, 2002). A range of pulmonary effects have been associated with PM exposure, including decreased lung development and function, exacerbation of asthma, allergy, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and increased risk of lung cancer (Alfaro-Moreno et al., 2007b; Borm and Donaldson, 2007; Kappos et al., 2004). The cardiovascular diseases associated with particle exposure include atherosclerosis, myocardial infarction and stroke (Bai et al., 2007; Schulz et al., 2005).

Despite the substantial amount of epidemiological data associating PM exposure with adverse health effects, the underlying biological mechanisms are not fully elucidated. Inflammation is involved in the development of many pulmonary and cardiovascular diseases, and inflammation provides a potential mechanistic link between PM exposure and adverse health effects (Alfaro-Moreno et al., 2007b; Bai et al., 2007; Donaldson et al., 2005; Frampton, 2006). The knowledge concerning the influence of particle source and physicochemical characteristics on the toxic effects of PM is also insufficient. WHO, therefore, recommends that all particles are considered to be equally hazardous per mass unit in current risk assessment. However, the evidence for an association with adverse health effects seems to be most consistent for PM emitted from the major mobile and stationary combustion sources (WHO, 2007). Toxicological and epidemiological research has also identified several particle characteristics, including size, transition metals and organic compounds, that may be associated with adverse health effects (Schwarze et al., 2006; WHO, 2007). Further research is, however, needed with respect to characterisation of the physicochemical properties and toxicity of source-specific PM.

1.1 Ambient particulate matter

1.1.1 Terminology

Ambient PM is a complex mixture consisting of solid particles and liquid droplets of varying size and composition. Airborne particles are classified by their aerodynamic properties since these properties govern the transport of particles in air, their deposition in the respiratory tract and to a certain extent reflect their chemical composition and source (WHO, 2000). The size of a particle is often described by its aerodynamic diameter, defined as the diameter of a unit density sphere with the same aerodynamic properties as the particle in question. The aerodynamic diameter of ambient particles varies from a few nanometres to tens of micrometers, and is highly dependent on physicochemical characteristics like shape, density and structure (Kreyling et al., 2007). Generally, only particles with aerodynamic diameters below 10 μm are considered to be relevant for health effects. During environmental monitoring, the mass concentrations of three size fractions of PM are commonly measured; coarse (aerodynamic diameters 2.5-10 μm), fine (0.1-2.5 μm) and ultrafine (<0.1 μm) particulate matter, and these are generally referred to as $\text{PM}_{2.5-10}$, $\text{PM}_{0.1-2.5}$ and $\text{PM}_{0.1}$ (Phalen, 2002).

1.1.2 Particle sources and composition

Ambient particles originate from three types of sources; (i) natural sources such as sea spray, soil erosion and forest fires, (ii) anthropogenic sources such as cars, planes, industry and residential heating or (iii) formation of secondary aerosols in the atmosphere by transformation of gases or vapours into liquids or solids. PM emitted from various sources differ considerably with respect to physical and chemical properties such as size, morphology, crystal structure, surface charge and chemical composition. Thus, the great diversity of particle sources causes the composition of ambient PM to be complex (Phalen, 2002). Environmental interactions with gases or sunlight may alter the physicochemical characteristics of the particles, in that photochemical processes lead to sulphur coating or modification of organic compounds (Paoletti et al., 2002; Paoletti et al., 2003; Vione et al., 2006). Ambient alterations of particles also include adsorption of biological material like pollen allergens or endotoxins from bacteria (Heinrich et al., 2003; Namork et al., 2006).

Traffic is considered to be a major particle source in most developed countries, both with respect to tailpipe emissions of combustion particles and resuspension of mineral particles from road abrasion (de Kok et al., 2006; Samet, 2007). Combustion particles from traffic are mainly carbon aggregates that consist of spherical primary carbon particles with diameters ranging from 20 to 50 nm (BéruBé et al., 1999; Dye et al., 2000; Paoletti et al., 2002). The small diameters of the primary particles provide a large surface area per mass, which allows for adsorption of various compounds such as metals, organic compounds, allergens and endotoxins (Figure 1). Thus, carbon aggregates may act as carriers that transport toxic or biologically active compounds into the lung. Mineral particles are arbitrarily shaped with larger diameters and therefore exhibit a smaller surface area per mass than carbon aggregates. The contribution from residential wood combustion to ambient particle concentrations is highly dependent on the season, but in the cold season wood smoke may contribute substantially to increased levels of air pollution locally, resulting in particle levels comparable to areas with a large traffic load (Glasius et al., 2006; Wu et al., 2007). Emissions from wood stoves generally consist of carbon aggregates, characterised by high levels of organic carbon (Dasch, 1982; Evans et al., 1981; Tesfaigzi et al., 2002).

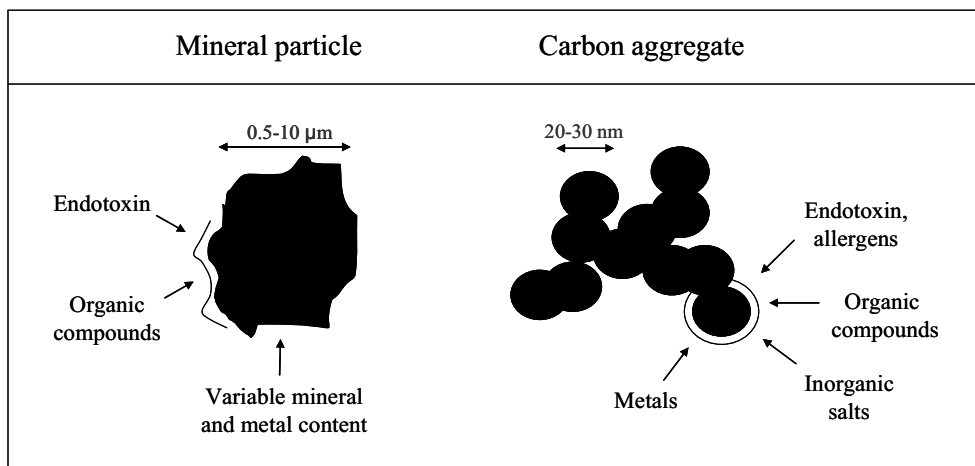


Figure 1: Schematic illustration of the differential characteristics of mineral particles and carbon aggregates.

1.2 Particle-induced health effects

1.2.1 Particle deposition and clearance

The principal function of the respiratory system is gas exchange through uptake of oxygen and excretion of carbon dioxide. The upper parts of the airway, the nasopharyngeal and tracheobronchial regions (Figure 2A), filter particulate matter and transport gas to bronchioles and finally to the alveoli where the gas exchange occurs. The adverse effects of inhaled PM are highly dependent on the deposition and retention of particles in the lung, and the deposition of particles is governed by their aerodynamic properties (section 2.1.1). In general, large particles primarily deposit in the nose and larger airways, whereas smaller particles pass through the upper airways and are deposited in the bronchial and alveolar regions (Figure 2B). Other particle characteristics such as shape and hygroscopicity also influence the aerodynamic properties of particles and thus their deposition site and probability in the lung (Kreyling et al., 2007; Löndahl et al., 2007). Moreover, particle deposition is highly non-uniform within regions of the lung, and some sites receive much greater particle doses than others. In the peripheral lung, particle deposition and retention is particularly high in the proximal alveolar region, which is defined as the area located in the transition between the terminal bronchiole and the alveolar space (Donaldson et al., 2008; Pinkerton et al., 2004; Saldiva et al., 2002).

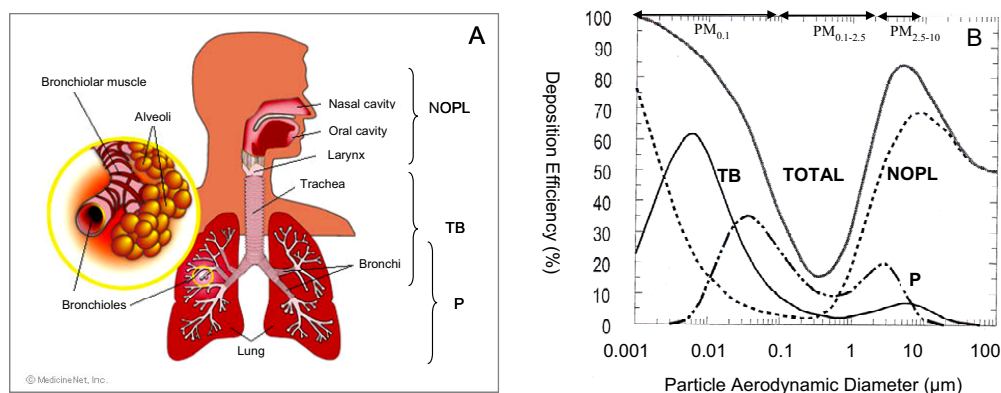


Figure 2:A) A schematic illustration of the human respiratory tract, divided into the nasopharyngeal region (nose mouth and throat, NOPL), the tracheobronchial region (TB) and the pulmonary or alveolar region (P) (modified from <http://www.associateprogramsales.com/Asthma/index3.html>). B) Particle deposition in the major regions of the human respiratory tract during normal respiration, corrected for the size-dependent inhalability (Phalen, 2002).

The deposition of particles in the lung is also influenced by the breathing patterns (e.g. sleep vs. exercise) and the geometry of lung (Kreyling et al., 2007). In some diseases such as asthma and COPD the geometry of the bronchial and alveolar regions may be changed and lead to a several fold increase in deposition of PM in the diseased parts of the lung (Chalupa et al., 2004; Kim and Kang, 1997).

Most particles deposited in the upper parts of the airway are transported with the mucus by ciliated cells to the back of the throat and swallowed. Although the majority of these particles are cleared within one day, some may penetrate the lung lining fluid and reach the ciliated cells, particularly in the bronchiolar region. The alveoli do not have ciliated cells, and particle clearance occurs by solubilization or phagocytosis by alveolar macrophages, followed by transport to the lymph system or to the throat by the ciliated cells (Phalen, 2002). Macrophage mediated particle removal is less efficient for ultrafine particles and may also be impaired in smokers, elderly and diseased subjects. Particles that are not cleared by macrophages may interact with epithelial cells, and there is increasing evidence for translocation of ultrafine particles across the epithelial barrier, into the blood stream and to secondary organs (Kreyling et al., 2007; Nemmar et al., 2002). Thus, the mechanisms for particle clearance depend on both deposition site and particle size. In addition, other physicochemical particle characteristics such as surface structure can also affect the phagocytosis of particles by macrophages and thereby the clearance mechanisms (Albrecht et al., 2007; Fang et al., 2006).

1.2.2 Alveolar cells and lung defence

The alveolar space is lined with epithelial cells which are classified as either type I or type II pneumocytes. Type I pneumocytes are large flattened cells (Figure 3), specialised for gas exchange, that cover more than 90 % of the alveolar air space but make up less than 10 % of the total alveolar cell number. The cuboidal type II pneumocytes are slightly more numerous but cover only 7 % of the alveolar surface. Type II pneumocytes produce surfactant and regulate the fluid balance. They are also involved in alveolar repair by replacement of injured type I cells through proliferation and differentiation (Fehrenbach, 2001; Steimer et al., 2005).

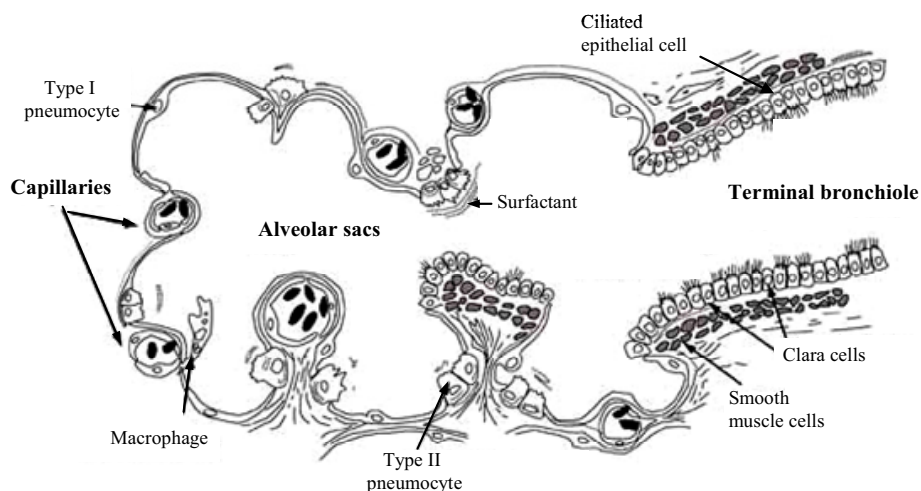


Figure 3: Schematic illustration of the alveolar region and its major cell types (modified from <http://www.lab.toho-u.ac.jp/med/physi1/respi/respi4,5/RH5-re.jpg>).

The gas exchange function of the alveoli depends on the thinness and integrity of the type I pneumocytes separating the air space from the pulmonary capillary. Deposited pathogens such as microorganisms or PM can damage the alveolar cells, and lung defence by innate and adaptive immunity is crucial to maintain normal lung function. The innate immune system provides an immediate but non-specific response, where resident macrophages are the first line of alveolar defence to deposited pathogens. Alveolar macrophages are mainly known as phagocytes that eliminate pathogens, but are also involved in maintenance and remodelling of lung tissue. In addition, both alveolar macrophages and type II pneumocytes contribute to immune regulation by secretion of a range mediators, including cytokines and growth factors (Mayer and Dalpke, 2007; Smith et al., 2007; Zhang et al., 2000).

The adaptive immune system requires recognition of specific antigens, such as a pathogen or its products, and may lead to development of immunological memory, providing a more efficient protection during future exposures to the same antigen (Curtis, 2005; Zhang et al., 2000). Lymphocytes and dendritic cells are the main mediators of the adaptive immune response which is essential for host defence against infections, but is also involved in allergic reactions. PM exposure may enhance allergic responses in animal models and in humans (Granum and Løvik, 2002; Saxon and Diaz-Sanchez, 2000), but the particle-induced effects on allergy are outside the scope of this thesis and will not be discussed further.

1.2.3 Inflammation

Inflammation is a critical step in the innate immune response. Its purpose is to remove the injurious stimuli and to initiate the healing process of the tissue. Inflammation is usually protective and beneficial, but has the potential to injure the airways (Larsen and Holt, 2000; Zhang et al., 2000). Acute inflammation is characterized by swelling, redness, pain, heat and loss of function, and is mediated through tissue infiltration by plasma and white blood cells such as neutrophils and monocytes. By initiation of an inflammatory response in the lung, neutrophils are recruited within hours after pathogen challenge. Neutrophils have a greater phagocytic activity than macrophages, and therefore contribute to enhanced phagocytic defense (Zhang et al., 2000). Monocytes are recruited to the inflammatory site within 24 to 48 hours (Larsen and Holt, 2000). Freshly recruited monocytes display a pro-inflammatory phenotype with high phagocytic activity, but during a few days they are differentiated to macrophages in the alveolar environment (Lambrecht, 2006). If the elimination of a pathogen fails, the acute inflammatory process could progress into chronic inflammation that might cause tissue damage. A chronic activation of the innate immune system could also induce a systemic inflammation, which includes increased levels of inflammatory mediators in the blood, activation and mobilization of inflammatory cells into the circulation and production of acute phase proteins in the liver (van Eeden et al., 2005).

Exposure to PM has been found to induce an influx of neutrophils and monocytes to the human lung (Ghio et al., 2000; Ghio, 2004; Salvi et al., 1999; Schaumann et al., 2004), suggesting that both cell types take part in particle-induced pulmonary inflammation. Systemic inflammation, with an increased mobilization of monocytes from the bone marrow, has also been reported in humans exposed to PM (van Eeden et al., 2001; van Eeden et al., 2005).

1.2.4 Inflammatory mediators

The recruitment and accumulation of inflammatory cells is orchestrated by a large number of inflammatory mediators, defined as chemical messengers that act on blood vessels and/or cells to produce an inflammatory response (Larsen and Holt, 2000; Zhang et al., 2000). Cytokines are a group of inflammatory mediators that are required in the initiation and progress of pulmonary inflammation. Pro-inflammatory cytokines such as tumour necrosis

factor (TNF)- α , interleukin (IL)-1, IL-6 and IL-8 may initiate and exacerbate inflammation, whereas anti-inflammatory cytokines like IL-4, IL-10 and IL-13 serve to reduce and regulate the inflammatory response and promote healing (Dinarello, 2000; Park and Pillinger, 2007). Additional negative regulation of the inflammatory response is provided by soluble cytokine receptors that bind and inactivate pro-inflammatory cytokines, such as IL-1 and TNF- α (Nicod, 1999).

IL-1 and TNF- α are early pro-inflammatory cytokines that initiate expression and release of a cascade of pro-inflammatory cytokines, including IL-1 and TNF- α themselves, but also other cytokines like IL-6, IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF), that contribute to the recruitment and activation of inflammatory cells (Chung, 2001; Dinarello, 2000; Driscoll, 2000; Kelly et al., 2003). The biological effects of TNF- α and IL-1 are very similar. However, TNF- α may elicit programmed cell death, whereas IL-1 β ¹ has been reported to inhibit apoptosis and augment alveolar epithelial repair (Coulter et al., 2002; Dinarello, 2000; Geiser et al., 2000). IL-8² is primarily known as a potent attractor and activator of neutrophils, but has also been found to exert chemotactic effects on several other cell types, including eosinophils, basophils and T-lymphocytes (Mukaida, 2003). IL-6 influences many aspects of immunity, it exerts effects on several immune cells, including increased antibody production in B-lymphocytes and proliferation, differentiation and cytokine release from T-lymphocytes. In addition, IL-6 induces systemic effects such as synthesis of acute phase proteins in the liver (Mills et al., 1999; Park and Pillinger, 2007), but can also act as an anti-inflammatory cytokine (Tilg et al., 1994).

Human particle exposure has been associated with increased levels of the pro-inflammatory cytokines IL-6 and IL-8 in the lung, and increased levels of IL-1 β , IL-6 and GM-CSF in the blood (Nordenhall et al., 2001; Rückerl et al., 2007; Salvi et al., 2000; van Eeden et al., 2001). Particle exposed macrophages and epithelial cells have also been reported to release a range of pro-inflammatory cytokines *in vitro*, such as TNF- α , IL-1 β , IL-6 and IL-8 (Dagher et al.,

¹ There are two functional forms of IL-1; IL-1 α and IL-1 β . In contrast to IL-1 β , IL-1 α is rarely secreted by cells and is active either as an intracellular molecule or as an integral membrane form (Dinarello, 1996). The effects of IL-1 α are not discussed further in this thesis.

² IL-8 has been renamed CXCL8, but the old nomenclature IL-8 is used in this thesis. IL-8 is a chemokine, which is defined as a cytokine that leads to cell migration, but will be referred to as a cytokine in the following.

2005; Ishii et al., 2004). Thus, exposure to PM seems to induce both pulmonary and systemic inflammation, reflected in increased levels of pro-inflammatory cytokines in the lung and in the blood.

1.2.5 Cell surface receptors

The pro-inflammatory cytokines IL-1 and TNF- α exert their cellular effects through binding to their respective transmembrane receptors, IL-1R and TNF-R. This initiates an intracellular signal transduction that results in translocation of transcription factors, such as nuclear factor (NF)- κ B. The signal transduction initiated by activation of IL-1R and TNF-R follow separate pathways, with receptor-specific molecules, but similar signalling principals apply. However, these signalling pathways converge on a common kinase complex that phosphorylates the NF- κ B inhibitory protein I κ B. This leads to degradation of I κ B and a subsequent release of NF- κ B, which is then translocated to the nucleus where inflammatory genes are expressed (Dinarello, 2000; Verstrepen et al., 2008). Toll like receptors (TLRs) are involved in the recognition of structurally conserved molecules from microbes, and belong to the same superfamily as IL-1R. Both the intracellular signalling and the cellular events initiated following activation of TLRs, IL-1R and TNF-R are very similar (Verstrepen et al., 2008). TLR4 recognizes lipopolysaccharide (LPS), a component of the outer cell membrane of Gram-negative bacteria, while TLR2 is involved in the recognition of components from Gram-positive bacteria such as peptidoglycan and lipoteichoic acid (Schwandner et al., 1999). Bacterial components may be bound to the surface of PM and contribute to the particle-induced inflammation. In human alveolar macrophages a TLR4 antagonist, but not a TLR2 antagonist, has been found to reduce the particle-induced cytokine release. In contrast, only the TLR-2 antagonist affected the cytokine release in normal human bronchial epithelial cells. This suggests that these two pulmonary cell types are activated by different bacterial components and through different receptors (Becker et al., 2005b; Becker et al., 2005a). In addition to interactions between particle bound bacterial components and TLRs, PM deposited in the lung may interact with a range of different receptors on the surface of pulmonary cells, including scavenger receptors, epidermal growth factor receptors or vanilloid receptors, depending on their physicochemical characteristics (Kobzik, 1995; Obot et al., 2002; Veronesi et al., 2002a; Wu et al., 2001).

1.2.6 Role of inflammation in particle-induced disease

Acute inflammation is a local protective reaction, whereas chronic inflammation can lead to a range of diseases, including pulmonary and cardiovascular diseases. The pulmonary diseases that have been linked to PM exposure, such as asthma, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and cancer, are all recognised as inflammatory diseases. Pulmonary fibrosis is believed to be related to a dysregulation in the communication between inflammatory and structural cells, mediated by various cytokines, chemokines and growth factors. IL-8 may be increased in patients with pulmonary fibrosis, but does not appear to be involved in the development of fibrosis (Kelly et al., 2003). On the contrary, TNF- α and IL-1 β have been identified as pro-fibrotic cytokines, and inhibition of these cytokines reduces silica-induced fibrosis in mice (Kelly et al., 2003; Rimal et al., 2005). Elevated levels of TNF- α , IL-1 β , IL-6 and IL-8 have been detected in sputum from COPD patients, and increased levels of IL-6 and IL-8 have also been associated with the severity of disease, measured as increased number of exacerbations (Chung, 2001). COPD leads to systemic inflammation, and these patients have an increased risk for cardiovascular disease (Sin and Man, 2007). TNF- α and IL-1 also seem to play a role in the pathogenesis of asthma, since they enhance the severity of asthma through lung inflammation (Berry et al., 2007; Kips, 2001). Furthermore, inflammation is considered to be a risk factor for most types of cancer, and the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-8 may be involved in the development of cancer through various steps of tumour formation, including cellular transformation, survival and proliferation (Aggarwal et al., 2006).

In the pathology of cardiovascular diseases, there is increasing evidence for local and systemic inflammation as a common mechanism (Kofler et al., 2005). Atherosclerosis is recognised as an inflammatory disease, and the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 are involved in induction of plaque destabilisation that can cause plaque rupture, thrombosis and lack of blood supply to the heart (ischemia) (Tousoulis et al., 2006). Circulating levels of TNF- α , IL-6 and IL-8 can also modulate cardiac contractility, and are often increased in patients suffering from chest pain due to lack of blood supply to the heart (unstable angina) or heart attack. The levels of these cytokines also seem to predict the risk for future cardiac events (Kofler et al., 2005; Tousoulis et al., 2006). Furthermore, systemic inflammation has been proposed as a mechanism for the impact of PM on the development of neurodegenerative conditions such as Alzheimer's disease (Calderón-Garcidueñas and Reed,

2007). Since the smallest ultrafine particles are preferentially deposited in the nasal region (Figure 2B) translocation of ultrafine particles to the brain via the olfactory nerve has been suggested as an alternative hypothesis (Calderón-Garcidueñas and Reed, 2007). Data concerning the ability of environmentally relevant particles to translocate from pulmonary tissues to the brain are, however, conflicting (Ghio and Bennett, 2007).

PM deposited in the lung may cause local and systemic inflammation through the release of pro-inflammatory cytokines, and a subsequent recruitment of inflammatory cells (Figure 4). The particle-induced inflammation is likely to cause a worsening of inflammatory diseases such as asthma, COPD and atherosclerosis. Therefore, inflammation is believed to play a key role in both pulmonary and cardiovascular diseases induced by PM (Alfaro-Moreno et al., 2007b; Bai et al., 2007; Donaldson et al., 2005). It should, however, be kept in mind that PM may also induce adverse health effects via inflammation-independent mechanisms. Other proposed mechanisms are translocation of particles to the blood or other organs (Bai et al., 2007; Calderón-Garcidueñas and Reed, 2007; Schulz et al., 2005), changes in cardiac rhythm caused by interaction with the nerve endings in the airway walls (Bai et al., 2007; Bérubé et al., 2007) and induction of DNA damage by the particle core or the adsorbed chemicals in the absence of inflammation (Schins and Knaapen, 2007).

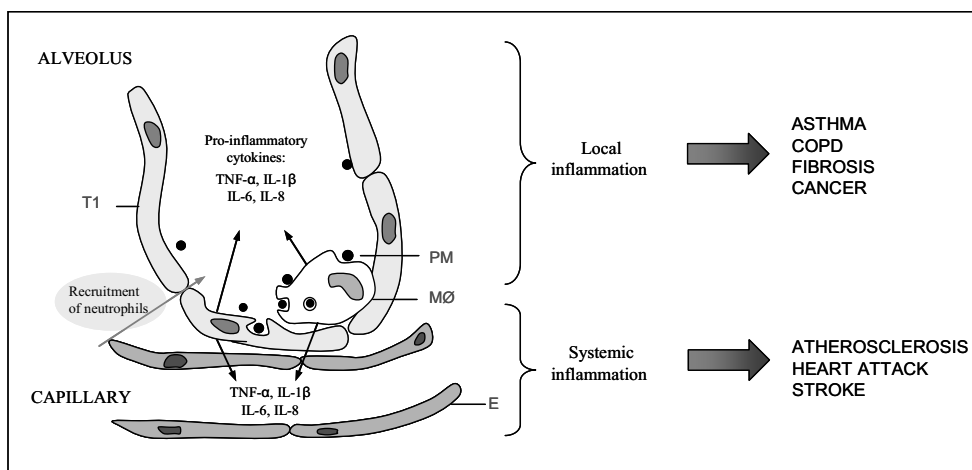


Figure 4: Illustration of how particle-induced inflammation may affect pulmonary and cardiovascular diseases. T1 = Type 1 pneumocyte, MØ = macrophage, E = endothelial cell, PM = particulate matter.

1.3 Role of physicochemical characteristics in particle-induced effects

Epidemiological and experimental studies provide increasing evidence for the importance of physicochemical characteristics in the particle-induced biological effects (Schwarze et al., 2006). A range of physicochemical characteristics that may influence particle-induced inflammation have been identified in *in vivo* and *in vitro* studies. The content of endotoxin, transition metals or various organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), nitro- and oxy-PAHs, have been reported to influence the particle-induced inflammation (Becker et al., 2005a; Ghio et al., 1999; Li et al., 2003b; Pagan et al., 2003; Schins et al., 2004; Schwarze et al., 2006; Xia et al., 2004), in addition to the surface charge and crystal structure (Albrecht et al., 2007; Sayes et al., 2006; Veronesi et al., 2002b). Moreover, small particles, exhibiting a large surface area per mass, have been found to induce a more pronounced pro-inflammatory response than larger particles of the same material. Both *in vitro* and *in vivo* experiments demonstrate that ultrafine carbon black and titanium dioxide particles are more potent in inducing inflammatory responses than the respective fine particles (Brown et al., 2000; Höhr et al., 2002; Monteiller et al., 2007; Murphy et al., 1999; Stone et al., 1998). Surface area has been suggested as a new dose metric for the inflammatory effects induced by these low-solubility low-toxicity particles *in vitro* and *in vivo* (Donaldson et al., 2008; Monteiller et al., 2007; Stoeger et al., 2006). In contrast, quartz is an example of a particle with a highly reactive surface that has been found to induce a much greater inflammatory response compared to the low-toxicity particles per unit surface area (Duffin et al., 2007; Monteiller et al., 2007). Thus, both surface area and surface reactivity should be considered as dose metrics for the inflammatory potential of particulate matter.

The influence of physicochemical characteristics on the pro-inflammatory response also varies between particles from different sources. For instance, the pro-inflammatory effects induced by diesel exhaust particles are mainly mediated by organic compounds, whereas transition metals account for the majority of the biological activity of residual oil fly ash (BéruBé et al., 2007; Ghio et al., 2002; Li et al., 2003a). Furthermore, different particle characteristics may be identified as explanatory factors depending on the choice of biological model system and the selection of biological parameters. This was recently demonstrated in a European multicentre project, RAIAP, comparing the inflammatory effects induced by PM from four European cities in different model systems. Responses in respiratory allergy models

were related to the organic markers, whereas inflammatory responses were associated with markers for crustal material (Steerenberg et al., 2006).

1.4 Particles from residential wood smoke and traffic

Epidemiological studies have associated exposure to traffic emissions with cardiovascular and pulmonary mortality, as well as morbidity-measures such as chronic bronchitis, respiratory symptoms and increased respiratory and cardiovascular hospital admissions (Hoek et al., 2002; Künzli et al., 2000; Samet, 2007). In contrast, the effects of exposure to wood smoke exposure on human health have not been well elucidated. However, two recent reviews conclude that particles from residential wood combustion seem to be equally harmful as particles from other sources, with the strongest association for pulmonary effects (Boman et al., 2003a; Naeher et al., 2007). Furthermore, a human inhalation study reported that wood smoke exposure affected both systemic and lung parameters, suggesting a potential impact of wood smoke particles on both pulmonary and cardiovascular diseases (Barregard et al., 2006; Barregard et al., 2008).

The negative effects associated with traffic exposure have been a major public health concern for several decades, and the physicochemical properties of traffic-derived particles have been characterised in numerous papers. The engines and the fuel composition influence the physicochemical characteristics of the emitted particles. Since these factors develop and change over time, the characteristics of emissions from on-road vehicles are not sufficiently described in the literature. Emissions from residential wood combustion have received less attention than traffic-derived particles, but the number of papers characterising wood smoke particles is currently increasing. Only one study compares the physicochemical characteristics of wood smoke and traffic-derived particles, and reports differences in the PAH profile, the particle size distribution and the elemental composition (Hedberg et al., 2002), that could possibly influence the biological effects induced by particles from the two sources. The inflammatory potential of particles from residential wood smoke compared to ambient traffic-derived particles has not been extensively investigated, but particles from both sources have been reported to induce inflammatory and toxic effects both *in vivo* and *in vitro* (Barregard et al., 2006; Barregard et al., 2008; Gerlofs-Nijland et al., 2007; Hetland et al., 2004; Jalava et al., 2007; Leonard et al., 2000; Seagrave et al., 2003; Tesfaigzi et al., 2002).

2 Aims of the study

The overall aim of the study was to characterise and compare particles from residential wood smoke and traffic with respect to physicochemical properties and pro-inflammatory potential. Moreover, the influence of the physicochemical particle characteristics on the pro-inflammatory response was investigated.

To achieve this, the following specific aims were set for the study:

1. To characterise the morphology and elemental composition of ambient air particles collected at sites dominated by residential wood smoke and traffic emissions, and to compare these with source-specific samples collected directly from the two combustion sources (Paper I).
2. To characterise wood smoke and traffic particles for use in biological experiments with respect to physical and chemical characteristics (Paper II).
3. To determine how wood smoke and traffic particles affected cell viability and release of pro-inflammatory mediators from a human monocytic cell line (THP-1) and a lung epithelial cell line (A549), with particular emphasis on the importance of physicochemical characteristics in the pro-inflammatory response (Paper III and IV).

3 Methodological considerations

3.1 Particle samples

For transmission electron microscopy (TEM) analysis of ambient particles from residential wood smoke and traffic, samples were collected in areas dominated by emissions from either one of the sources (Wood 1 and 2, Vehicle 1 and 2). The source contributions to these ambient samples were verified by comparison with source-specific (Sp) samples, collected

Table 1: Particle samples included in the study

Paper	Sample	Description of site or source	Relation to samples used in other papers
Paper I			
<i>Ambient or source specific (Sp) samples, collected for characterisation in TEM.</i>	Vehicle 1	Road tunnel	
	Vehicle 2	Highway intersection	
	Vehicle Sp1	Diesel vehicle	
	Vehicle Sp2	Gasoline vehicle	
	Wood 1	Farmhouse	
	Wood 2	Residential area	
	Wood Sp1	High-temperature combustion (T \approx 1000 ° C)	
	Wood Sp2	Low-temperature combustion (T \approx 500 ° C)	
Paper II + III			
<i>Samples collected for biological experiments.</i>	Tunnel St+	Road tunnel (winter season, studded tires)	Same site as Vehicle 1 (Paper I)
	Tunnel St-	Road tunnel (summer season, normal tires)	
	Wood	High-temperature combustion	Same site and similar combustion conditions as Wood Sp1 (Paper I)
	Diesel	Industrial forklift SRM2975	
	Porphy	Laboratory generated	
Paper IV			
	Road tunnel	Road tunnel (without studded tires)	= Tunnel St- (Paper II + III)
	Traffic	Highway intersection	Same site as Vehicle 2 (Paper I)
	Wood	High-temperature combustion	= Wood (Paper II + III)
	Diesel	Heavy duty diesel vehicles, early 1980s (SRM1650a)	
	Quartz	Laboratory generated (Min-U-Sil)	

directly from relevant sources; a diesel car, a gasoline car and high- and low-temperature wood combustion (Table 1). A high-volume sampler was not available for collection of sufficient amounts of ambient air particles for biological experiments. Traffic samples were, therefore, collected in a road tunnel where the particle concentrations were high, whereas wood smoke particles were sampled directly from a conventional wood stove during high-temperature combustion (Paper II). Wood smoke particles from high- rather than low-temperature combustion were collected for biological experiments, since the morphology of particles from low-temperature combustion (large spherical carbon particles, Figure 6) was not observed in the ambient wood smoke samples (Paper I).

Vehicles are known to contribute to ambient particle levels both through tailpipe emissions (combustion particles), abrasion of road pavement (mineral particles) and wear particles from brakes and tires (Adachi and Tainosho, 2004; de Kok et al., 2006; Furusjo et al., 2007; Samet, 2007). Human exposure to combustion particles from vehicles is likely to be accompanied by exposure to mineral particles from road abrasion and various levels of other wear particles depending on driving pattern (stop/start vs. continuous). The tunnel samples and the ambient traffic sample, that contained both combustion and mineral particles, were therefore considered to be representative for traffic-derived particles in the biological experiments (Paper III and IV). Tunnel St+ was collected during the winter season, when studded tires, equipped with metal studs to avoid sliding on icy road surfaces, were in use. Compared to tires without studs, the studded tires cause more road abrasion and thereby greater concentrations of mineral particles (Kupiainen et al., 2003). To investigate the relative importance of mineral and combustion particles in the pro-inflammatory response to traffic-derived particles, samples containing only mineral particles or traffic-derived combustion particles were included as model particles in the biological experiments (Paper III and IV). The model particles Porphyr and Diesel induced a negligible response in a monocytic cell line (Paper III). To investigate if these low responses were representative for other samples containing only mineral particles or carbon aggregates, samples that had previously been found to induce a greater pro-inflammatory response were used in Paper IV ((Øvrevik et al., 2005), personal communication R.B. Hetland, Norwegian Institute of Public Health, Oslo, Norway). In addition, Tunnel St+ was replaced by an ambient traffic sample (see Table 1 for an overview of the particle samples).

All the traffic-derived samples used in biological experiments contained contributions from a wide range of on-road vehicles, including cars, trucks and buses fuelled with diesel or gasoline (Paper III and IV), and were therefore likely to have similar characteristics as the traffic-particles inhaled during ambient exposures. The conventional wood stove chosen for particle collection was considered to be representative for the stoves and boilers accounting for the majority of the particle emissions in the Nordic countries (Denmark, Finland, Norway and Sweden), since it had similar combustion technology (Sternhufvud et al., 2004). However, the physicochemical characteristics of wood smoke particles vary considerably with combustion temperature and air supply (Boman et al., 2003a; Pagels et al., 2006; Rau, 1989). The dominating chemical characteristics range from sugars and methoxyphenols at low temperatures (<700 °C) to hydrocarbons like PAHs and benzene at medium temperatures (700-900 °C) to alkali salts at high temperatures (>900 °C) (Johansson et al., 2003; Kjällstrand and Olsson, 2004). Since ambient wood smoke particles originate from a range of combustion conditions, Wood that was collected during high-temperature combustion (Table 1) had certain limitations with respect to relevance for ambient exposures. To confirm that the biological effects observed during exposure to Wood were also representative for wood smoke particles from other combustion conditions, additional experiments were performed in a subsequent study with samples collected during different phases of the combustion cycle, which included particles emitted from a wider range of combustion conditions (Schwarze et al., 2008)(for stove details, see Sällsten et al. (2006)).

3.2 Sampling methods

A variety of sampling devices may be applied to collect aerosols for investigation of particle-induced health effects. The collection of PM for different purposes, such as physicochemical characterisation or biological experiments, generally requires different sampling strategies. In the present study, a Respicon® virtual impactor loaded with polycarbonate membranes was used to collect particles for TEM analyses. Respicon® separates particles into three size fractions, but since the analysis of combustion particles was the main area of interest, only the PM_{2.5} fraction was analysed (Paper I). An advantage by collection of particles on polycarbonate membranes is that they allow for TEM analyses without particle extraction, since carbon or germanium extraction replicas of the membranes can be obtained.

Polycarbonate membranes were also applied for collection of particles for biological experiments. Since we did not have access to a high-volume sampler, two different sampling devices, described in Paper II, were used to collect particles on a series of polycarbonate membranes to obtain sufficient amounts of PM. The particles were then scraped of the filters and pooled (Paper II). A major disadvantage by collecting PM with high volume impaction or filtration is that vigorous methods are necessary to remove particles from the filters. This is most commonly achieved by extraction with a solvent such as ultra pure water or methanol, combined with sonication. These procedures may, however, alter the physical and chemical characteristics of the collected PM (Ayres et al., 2008; Bérubé et al., 1999). Although a more gentle procedure was used to remove the particles from the polycarbonate membranes in the present study, the particles were similarly pre-treated, first with methanol to inactivate fungi and then by sonication to suspend particles in medium for biological experiments. A promising new method has, however, been developed to overcome the disadvantages associated with filtration and impaction; collection of particles directly into a fluid. Apparently, this collection method does not influence particle properties such as size, bulk chemistry or single particle chemistry and morphology (Ayres et al., 2008; Kim et al., 2001).

The samples collected for biological experiments were considered to be total suspended particulate matter (TSP), since the two sampling devices used in Paper II did not have a well defined cut-off size. The TEM analyses did, however, show that the majority of the particles were below 10 μm , and thus belonged to the respirable particle fraction.

3.3 Physicochemical particle characterisation

To be able to target environmental risk reduction strategies it is crucial to identify the physical and chemical properties that determine PM toxicity (WHO, 2007). In toxicological studies, particle characterisation is often combined with statistical analyses such as uni- or multivariate regression analysis, to investigate the influence of particle properties on inflammation and toxicity. Another strategy is to separate particle samples into different fractions by organic or aqueous extraction to identify the most potent fraction. Alternatively, various inhibitors may be used to investigate the influence of specific particle components such as metals or endotoxin on the biological response. In the present study, these different approaches were combined to investigate the influence of the physicochemical characteristics on the pro-inflammatory potential of wood smoke and traffic-derived particles.

Generally, the characterisation of PM may be divided into bulk chemical analysis and single particle analysis (Pooley and de Mille, 1999). Bulk analyses are performed on PM collected on a filter substrate, and provide information about the chemical composition, such as content of organic or inorganic species, per PM mass concentration (Pooley and de Mille, 1999). Physical properties, such as surface area may also be measured after particle collection by gas adsorption techniques such as BET³ measurements (Wittmaack, 2007). In addition, a large number of instruments provide time-resolved analysis of physical and chemical particle characteristics on a time scale ranging from seconds to hours (McMurry, 2000). PM samples used in biological experiments are, however, usually collected over days or weeks, and the time-resolved particle characteristics may be difficult to relate to the composition of the samples collected for biological experiments.

Single particle analysis generally refers to application of electron microscopic techniques, such as scanning or transmission electron microscopy (SEM, TEM), x-ray microanalysis (XRMA), selected area electron diffraction (SAED) and electron energy loss spectroscopy (EELS). These techniques provide information about the size, morphology, elemental composition and structure of individual particles (BéruBé et al., 1999; Buffat, 1999; Casuccio et al., 2004; Maynard, 2000; Pooley and de Mille, 1999). Although single particle analysis provides a higher level of detail, application of bulk analyses may be equally important to measure chemical characteristics that could affect particle-induced health effects.

3.3.1 Morphology

To identify different particle classes in ambient and source-specific samples, the morphology and the elemental composition of individual particles was analysed by TEM and XRMA (Paper I). For particles with large variations in chemical composition, structure, surface area and solubility, such as the particle classes identified in Paper I, other particle properties than morphology are more likely to determine the pro-inflammatory potential. Therefore, a detailed morphological characterisation, such as image analysis of TEM images to determine shape factors (e.g. particle area, breadth, length, perimeter (BéruBé et al., 1999)), was not

³ BET refers to the initials of Stephen Brunauer, Paul Emmet and Edward Teller who developed the theory that this gas adsorption method is based on.

performed. Particle morphology was, however, used to estimate the content of carbon aggregates and mineral particles in samples collected for biological experiments (Paper II).

3.3.2 Primary particle diameter

The diameters of primary particles in carbon aggregates from wood smoke and traffic were measured by TEM as an indicator of surface area per mass (Paper I and II). In toxicological studies, surface area has either been calculated from the mean primary particle diameter (Brown et al., 2001; Höhr et al., 2002; Murphy et al., 1999) or measured by gas adsorption (Hetland et al., 2001; Lison et al., 1997; Monteiller et al., 2007). Since surface area measurements based on these two methods have been found to correlate for particles larger than 20 nm (Wittmaack, 2007), primary particle diameter was considered to be a suitable indicator for particle surface area in the present study.

3.3.3 Elemental composition

XRMA was used to study differences in the elemental composition of carbon aggregates from wood smoke and traffic (Paper I). Several elements, Ge, Cu, V, Cr, Fe and Co, were detected in the background XRMA spectra due to contributions from the microscope column and the specimen holder or support. It was, therefore, not possible to determine if these elements were present in the carbon aggregates. Moreover, some elements, Al, P, Cl and Zn, were only detected occasionally in carbon aggregates, but might be present in large enough amounts in bulk samples to have biological relevance. The elements detected most frequently in the carbon aggregates, Si, S, K and Ca, have not been associated with particle-induced inflammation *in vitro* (Paper I), and XRMA of carbon aggregates was, therefore, not performed on the samples collected for biological experiments (Paper II).

3.3.4 Carbon and PAH analyses

The influence of the organic carbon content and the sum of the 18 measured PAHs on the cytokine release was investigated by linear regression analysis (Paper II and III). In addition, organic extraction was used to investigate the role of the organic fraction in the pro-inflammatory response by comparison of the response to native particles, organic extracts and washed particles (Figure 5). Dichloromethane (DCM) was used for organic extraction in

Paper III, but was for practical reasons replaced by methanol in Paper IV. The difference in polarity between these two solvents could cause extraction of different selections of organic compounds. However, extracts made with the two solvents induced a similar release of pro-inflammatory mediators in the co-culture system (data not shown), suggesting that the organic compounds involved in the pro-inflammatory response were extracted to a similar extent by the two solvents.

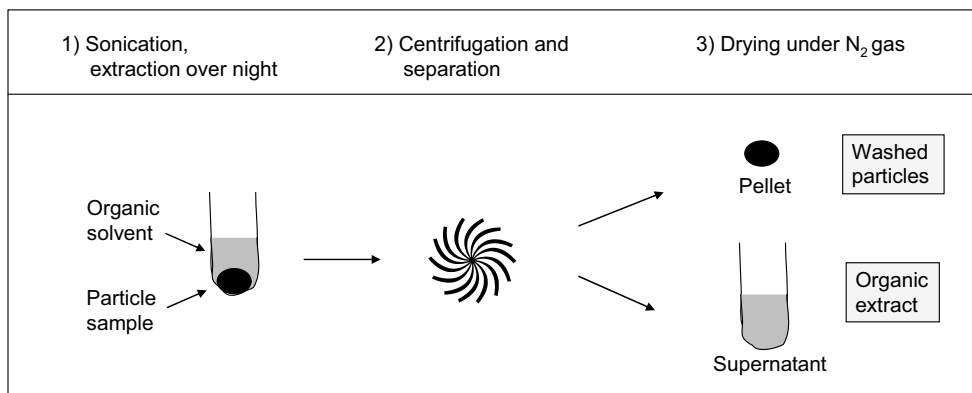


Figure 5: Illustration of the extraction procedure for washed particles and organic extracts.

3.3.5 Endotoxin

Particles collected in ambient environments may be contaminated with microbial endotoxin. The traffic-derived particles interacted with ambient or tunnel air prior to collection, whereas wood smoke particles were collected directly from the wood stove. A difference in endotoxin content was therefore likely to reflect a difference in sampling conditions (ambient vs. laboratory) rather than a difference between the two sources. The influence of particle-bound endotoxin on the pro-inflammatory mediator release was investigated using the endotoxin inhibitor polymyxin B sulphate (Paper III and IV). In addition, the endotoxin content in aqueous particle extracts was analysed by kinetic limulus amoebocyte lysate (LAL) analysis (Kinetic-QCL[®], Cambrex, Walkersville, MD, USA). However, the endotoxin content measured by LAL did not correlate with the reduction in cytokine release induced by the endotoxin inhibitor polymyxin (Paper III and IV). A possible explanation could be that only the water soluble endotoxin was measured by the LAL technique, whereas particle bound endotoxin also is likely to be potent in interaction with cells. We chose to rely on the polymyxin data, rather than the LAL measurements, since some studies suggest that the

reduction in cytokine release induced by inhibition with polymyxin provides a more accurate measure of endotoxin in particle samples (Alexis et al., 2006; Soukup and Becker, 2001). Polymyxin also has certain limitations, in that it only inhibits the cellular effects of lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria, while other bacterial components might also induce cytokine release (Becker et al., 2002; Soukup and Becker, 2001). An alternative approach could be to apply inhibitors of TLR2 and TLR4, which are involved in recognition of bacterial components from Gram-positive and Gram-negative bacteria, respectively (Becker et al., 2002; Becker et al., 2005a). However, particulate matter without endotoxin has been suggested to induce inflammatory responses through interaction with TLR4 (Cho et al., 2005; Karimi et al., 2006), whereas endogenous stimuli such as oxidative stress and necrotic cells seem to induce a TLR2 dependent expression of inflammatory genes (Beg, 2002; Kirschning and Schumann, 2002). Thus, a possible disadvantage by application of these antagonists is an overestimation of the influence of bacterial components on the inflammatory response.

3.4 Biological model systems

Cell lines are useful biological model systems for investigations of the underlying mechanisms of particle-induced effects and for rapid screenings of many particle samples or components of complex mixtures. Compared to primary cells, cell lines are homogeneous and stable and therefore provide higher reproducibility. Furthermore, experiments in cell lines are inexpensive and can be performed rapidly and be replicated in multiple laboratories. However, these cells have changed features in comparison to normal cells in tissue, and they retain little phenotypic variation (Devlin et al., 2005; Rothen-Rutishauser et al., 2007; Steimer et al., 2005).

A major limitation of *in vitro* cell models in investigations of particle-induced effects is that the particle exposure does not mimic the conditions during *in vivo* exposures. These models also lack the cellular interactions and neurological signals that are of importance in animals, and results obtained from *in vitro* studies should, therefore, not be extrapolated to the *in vivo* situation (Devlin et al., 2005). In animals or humans, the toxic effects are not limited to expression of signalling molecules and changes in cell viability, but include migration of inflammatory cells, changes in the vascular compartment, tissue injury and fibrotic alterations. Results from *in vitro* studies should, therefore, be confirmed in *in vivo* models,

since these are more suitable for prediction of pathological events in the human lung (Maier et al., 2008). We did not have the opportunity to perform *in vivo* studies, and must therefore rely on experimental and epidemiologic studies in the literature to demonstrate the relevance of our *in vitro* results.

In a healthy lung, the epithelial cells lining the alveoli and the resident macrophages are primary cellular targets for deposited particles. Monocytes have, however, been found to accumulate in the alveoli during lung inflammation (Goto et al., 2004a; Maus et al., 2001; Rosseau et al., 2000), and have been suggested to play a role in particle clearance (Goto et al., 2004b). The monocytic cell line THP-1 (peripheral blood, leukaemia) was therefore used as a model system for alveolar mononuclear phagocytes during acute lung inflammation (Paper III). This model system was then improved by expansion into a contact co-culture of THP-1 monocytes and A549 pneumocytes (Paper IV). The A549 cells (lung carcinoma) exhibit a phenotype that has some similarities to primary alveolar type II pneumocytes, and are commonly used as an *in vitro* model for assessment of pulmonary toxicity (Steimer et al., 2005). A major advantage by application of co-cultures rather than mono-cultures is that they allow for some cellular communication, which has been found to influence the particle-induced release of pro-inflammatory mediators (Drumm et al., 2000; Jimenez et al., 2002).

3.4.1 Particle concentrations

For non-adherent cells, the particle concentration per volume is the most relevant exposure measure, whereas the concentration of particles per surface area is more relevant for adherent cells. Therefore, particle concentrations were expressed in $\mu\text{g/ml}$ when applied to the non-adherent THP-1 monocytes (Paper III), and in $\mu\text{g/cm}^2$ when applied to the co-culture which included the adherent A549 pneumocytes (Paper IV). The particle concentrations applied in Paper III and IV are listed in both units in Table 2. In order to investigate the influence of different particle characteristics on the pro-inflammatory response, we used relatively high particle concentrations in our experiments. The applied particle concentrations are much higher than the average particle deposition on the lung surface during normal ambient concentrations. However, the deposition of particles in the human airway is very uneven, and particle retention for fine and ultrafine particles has been suggested to be highest in the proximal alveolar region (Donaldson et al., 2008; Pinkerton et al., 2004; Saldiva et al., 2002). In addition, particle clearance is slow in the alveolar region, and biological half-lives for

alveolar clearance have been found to be as high as 120 days in young healthy non-smokers and even higher for elderly, smokers and diseased subjects (Möller et al., 2001). Thus, the particle concentrations used in the present study might be relevant for specific regions of the lung during long time exposure to high concentrations of air pollution. Application of high particle concentrations may be useful in *in vitro* studies that aim to investigate the mechanisms of toxicity, such as the present investigations on the influence of different particle characteristics on the pro-inflammatory response. These data should, however, not be extrapolated for risk assessment purposes.

Table 2: Conversion between the two units used to describe particle concentrations in biological experiments (Paper III and IV). Concentrations were expressed in $\mu\text{g}/\text{ml}$ in Paper III and $\mu\text{g}/\text{cm}^2$ in Paper IV.

Paper III		Paper IV	
Monoculture of THP-1 monocytes		Co-culture of THP-1 monocytes and A549 pneumocytes	
$\mu\text{g}/\text{ml}$	$\mu\text{g}/\text{cm}^2$	$\mu\text{g}/\text{ml}$	$\mu\text{g}/\text{cm}^2$
30	5	40	10
70	12	80	20
140	24	160	40
210	36		
280	48		

3.4.2 Cell viability

Cell viability was determined by counting the proportion of trypan blue-negative cells in a Bürker chamber. For unexposed cells, the number of viable cells showed a time dependent increase, reflecting cell proliferation. In contrast, the cell numbers did not increase with time during exposure to some particle samples, but were significantly lower than in unexposed controls. The reduction in cell numbers could be due to necrosis, apoptosis or decreased proliferation. In order to investigate the influence of necrosis and apoptosis on the reduction in number of viable cells, the particle-induced necrosis/cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) from the cytosol of damaged cells into the cell culture medium, whereas the percentage of apoptotic cells was determined by Hoechst 333342/propidium iodide staining and flow cytometry ((Låg et al., 2002), Paper IV). The reduction in cell numbers could not be explained by necrosis or apoptosis, suggesting that the reduction was due to a reduced proliferation. Since particle induced effects on proliferation were not measured by a conventional method, such as thymidine incorporation, the effects will be referred to as a reduction in cell number rather than reduced proliferation.

4 Summary of papers and results

4.1 Papers

The present study consists of four papers that compare the physicochemical characteristics of particles from residential wood smoke and traffic, and investigate how particle characteristics may affect the pro-inflammatory potential of particles from the two sources. To compare the physicochemical particle characteristics, samples were collected at sites dominated by either one of the two sources, as ambient particles were considered to be most relevant for human exposures (Paper I). Ambient samples could not be obtained for biological experiments, since a high volume sampler was not available, but were collected directly from the combustion source (wood stove) or at a site with particularly high particle concentrations (road tunnel). The latter samples were analysed in Paper II in order to investigate if the physicochemical

Table 3: The applied methods and the respective measured parameters are listed for the separate papers (for abbreviations used, see page 5). The analysed particle samples are described in Table 1.

Paper	Method	Measured parameter
Paper I		
<i>Ambient and source-specific samples</i>	TEM	Particle morphology Primary particle diameter
	XRMA	Elemental composition
Paper II		
<i>Samples collected for biological experiments</i>	TEM	Particle morphology
	TEM	Primary particle diameter
	HR-TEM	Microstructure
	EELS/SAED	Graphitic character
	TOT	Elemental, organic and total carbon
	GC-MS	PAH
Paper III §		
<i>Monoculture</i>	ELISA*	IL-1 β , TNF- α , IL-8
<i>THP-1 cells</i>	Cytotoxicity detection kit	LDH
<i>(2, 5, 12 h exposure)</i>		
Paper IV		
<i>Contact coculture,</i>	ELISA*	TNF- α , IL-6, IL-8
<i>THP-1 + A549 cells</i>	Cytotoxicity detection kit	LDH
<i>(12, 40, 64 h exposure)</i>	Trypan blue exclusion	Number of viable cells

§ Real-time PCR was performed in this paper, and increased levels of IL-1 β , TNF- α , IL-8 and IL-10 mRNA were detected. Since the measurements were not repeated in Paper IV and this analysis only was a minor part of Paper III, the data are not discussed in this thesis.

* Only cytokines that increased during particle exposure are listed.

characteristics of particles from wood smoke or traffic influenced the pro-inflammatory responses detected in Paper III and IV. All the methods are listed in Table 3, together with the respective measured particle characteristics. The pro-inflammatory effects of particles from wood smoke and traffic were investigated in two *in vitro* models; a monoculture of THP-1 monocytes (Paper III) and a contact co-culture of THP-1 monocytes and A549 pneumocytes (Paper IV). In Paper IV, the study design was further improved by increasing the maximum exposure time from 12 h to 64 h and measuring the number of viable cells.

4.2 Results

Transmission electron microscopy (TEM) of samples collected directly from a wood stove showed that the morphology of wood smoke particles varied with the combustion conditions. Non-aggregated large spherical carbon particles (diameter 50-600 nm) were emitted from low-temperature combustion, while aggregates of primary carbon particles (20-40 nm) with branched morphology were emitted from high-temperature combustion (Figure 6a and b, respectively). In ambient samples, collected in areas dominated by either wood smoke or traffic emissions, carbon aggregates were the only combustion particles observed, indicating that this was the predominating morphology of combustion particles in ambient air (Paper I). In samples collected for biological experiments, in traffic-dominated areas or directly from a wood stove, carbon aggregates were also the only combustion-derived particles observed. The traffic-derived samples contained mineral particles originating from road abrasion (Figure 6c, arrow) and iron particles from brake wear, in addition to carbon aggregates. Furthermore, the content of mineral particles was higher in the in the season when studded tires were used as compared to when normal tires were used (Paper II).

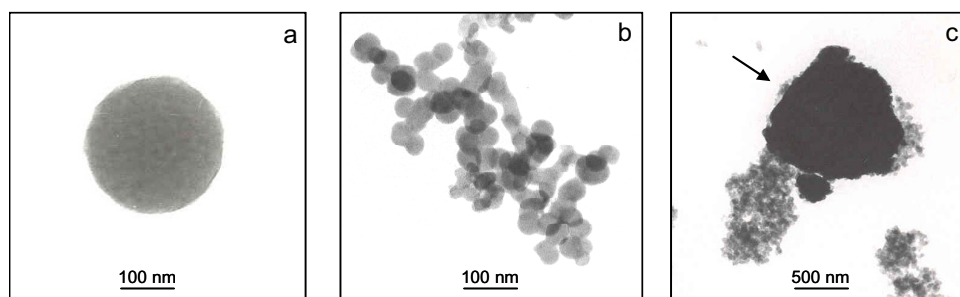


Figure 6: TEM micrographs of (a) large single spherical carbon particle, (b) carbon aggregate from wood smoke and (c) carbon aggregates and mineral particle (arrow) from road tunnel. The details of TEM instruments are given in Paper I.

The elemental composition of single carbon aggregates was studied by X-ray microanalysis (XRMA), where C and O were identified as the most abundant elements. Carbon aggregates from wood smoke were, in addition, characterised by traces of K, while carbon aggregates from traffic were characterised by Si and Ca (Paper I). The primary particles in carbon aggregates from traffic (24 ± 7 nm) were significantly smaller than in wood smoke aggregates (31 ± 7 nm), indicating a larger surface area for combustion particles emitted from traffic (Paper I and II). The primary particles of carbon aggregates have been reported to exhibit an internal turbostratic microstructure, consisting of a concentric arrangement of layer planes with a two dimensional graphitic structure, lacking the ordered stacking of graphite, and thus its three dimensional structure (Heckman, 1964). The turbostratic microstructure of primary particles from traffic and wood smoke was found to differ in that concentric carbon layers surrounding several nuclei were observed in traffic-derived particles as opposed to a single nucleus in wood smoke (Paper II, Figure 2). In contrast, the graphitic character, defined as the degree of similarity to the structure of graphitic carbon, did not differ between the two sources. Furthermore, both the total PAH content measured as the sum of 18 PAHs and the content of organic carbon was higher in wood smoke as compared to traffic-derived particles (Paper II).

Carbon aggregates from wood smoke and traffic differed with respect to elemental composition, primary particle diameter and turbostratic microstructure. Furthermore, the samples collected from these two sources differed with respect to the content of organic compounds, measured as the sum of 18 PAHs and the content of organic carbon. However, since it is crucial that the physicochemical characterisation is performed on the same samples as used for biological experiments, only the data from Paper II could be applied to investigate the influence of physicochemical characteristics on the inflammatory potential of the particles.

Particles from wood smoke and traffic induced a similar pro-inflammatory response after 12 h exposure in both *in vitro* model systems, with respect to release of TNF- α , IL-6 and IL-8 (Paper III and IV). However, the traffic-derived particles, but not wood smoke, induced a release of IL-1 β from the monocytic cell line (Paper III). In the co-culture, particles from the two sources were also found to induce differential response patterns for increasing exposure times, 40-64 hours, as the traffic-derived particles induced a greater release of IL-6 and IL-8

and a less pronounced reduction in the number of viable cells as compared to wood smoke (Paper IV). The cytotoxicity was generally low for all the included particle samples in both model systems. The influence of IL-1 and TNF- α on the particle-induced release of IL-6 and IL-8 was studied in the co-culture by application of antagonists. The influence of TNF- α on the cytokine release did not differ between particles from the two sources, while IL-1 affected the release of IL-6 during exposure to the traffic-derived particles, but not to wood smoke (Paper IV). This indicates a more pronounced role for IL-1 in the early pro-inflammatory response to traffic-derived particles, in line with the observation of an IL-1 β release from the monocytic cell line only during exposure to the traffic-derived particles. These results suggest a source-dependent difference in pro-inflammatory response patterns, since traffic-derived particles induced a greater release of IL-1 β , IL-6 and IL-8, whereas wood smoke particles caused a more pronounced reduction in the number of viable cells.

With respect to the influence of physicochemical characteristics on the pro-inflammatory response, the release of pro-inflammatory mediators did not increase with increasing surface area for washed particles of Wood and Diesel, the samples containing carbon aggregates only. This indicated that other particle properties than the surface area per mass determined the pro-inflammatory potential of these particles (Paper III). Linear regression analysis, however, suggested that both the content of organic carbon and the total PAH content influenced the particle-induced cytokine release (Paper III). The PAH contents in the organic extracts were estimated by assuming that all the PAHs were extracted, and were found to be 100-150 times higher in extracts from wood smoke than from traffic-derived particles. The biological responses induced by the organic wood smoke extracts were, however, never more than twenty times higher than the response induced by the organic extracts of traffic-derived particles (cytokine release or reduction in cell number, Paper III and IV). This suggests that other organic compounds, in addition to PAHs, were involved in the pro-inflammatory response to wood smoke and traffic-derived particles.

The release of pro-inflammatory mediators induced by particles from the two sources was mediated by different particle fractions. The organic fraction of wood smoke particles accounted for the majority of the cytokine release, whereas the washed particle fraction had a stronger influence on the cytokine release induced by traffic-derived particles (Paper III and

IV). The reduction in the number of viable cells was, however, associated with the organic fraction for both sources (Paper IV).

5 Discussion

Particle emissions from residential wood combustion and traffic contribute substantially to ambient particle levels in many industrialised countries (de Kok et al., 2006; Saarikoski et al., 2008; Song et al., 2007; Wu et al., 2007). Whereas exposure to traffic-derived particles occurs throughout the year, wood smoke exposure is seasonal, but the contributions from residential wood combustion and traffic to urban air have been reported to be of a similar magnitude in the cold season (Glasius et al., 2006; Saarikoski et al., 2008; Song et al., 2007; Wu et al., 2007). Exposure to PM from both sources has been associated with adverse health effects in epidemiological studies, and particles from wood combustion, diesel vehicles and road traffic (exposure in a road tunnel) have also been found to induce inflammatory responses in human inhalation studies (Barregard et al., 2006; Barregard et al., 2008; Boman et al., 2003a; Larsson et al., 2007; Salvi et al., 1999; Salvi et al., 2000; Samet, 2007). The relative inflammatory potential of particles from wood smoke and traffic has, however, not been extensively investigated. In the present study, particles from the two sources induced a similar acute pro-inflammatory response *in vitro* after 12 hours, but for increasing exposure times, 40-64 hours, particles from wood smoke and traffic showed differential response patterns. Traffic-derived particles generally induced a greater cytokine release and a less pronounced decrease in the number of viable cells compared to wood smoke particles. In accordance with the present results, emissions from these two combustion sources have been reported to induce different effects on lung inflammation in male rats, since wood smoke particles induced greater levels of TNF- α and a higher increase in macrophage numbers and markers for lung toxicity in lung lavage fluid, whereas diesel exhaust particles induced higher levels of MIP-2, the rat analogue of IL-8 (Seagrave et al., 2005).

5.1 Physicochemical characteristics and their influence on the pro-inflammatory response

Particulate matter (PM) may differ considerably with respect to both chemical composition and physical properties such as size, morphology and structure, and these physicochemical characteristics may be of importance for particle-induced health effects (de Kok et al., 2006; Dreher, 2000; Gonzalez-Flecha, 2004; Schlesinger et al., 2006; Schwarze et al., 2006). The relative importance of physicochemical characteristics in the inflammatory response to wood smoke and traffic-derived particles is, however, still under investigation.

5.1.1 Morphology

Carbon aggregates were the only combustion particles detected in the ambient samples collected in areas dominated by residential wood smoke or traffic emissions. This suggested that carbon aggregates was the dominating morphology of combustion particles in ambient air (Paper I). In accordance with this, carbon aggregates have been reported to be the most abundant combustion particles in urban areas (Paoletti et al., 2002; Shi et al., 2003), although spherical fly ash particles with diameters ranging from 100 nm to several micrometers may contribute considerably in some residential and industrial areas (Jones et al., 2006; Lewis et al., 2003; Shi et al., 2003).

The morphology of residential wood smoke particles in ambient air has not been described previously, but several studies describe the morphology of particles collected directly from wood stoves (Colbeck et al., 1997; Hallett et al., 1989; Mavrocordatos et al., 2002; Pagels et al., 2006). Carbon aggregates, have been observed in emissions from a range of combustion conditions, in samples from conventional wood stoves (Tesfaigzi et al., 2002), open fireplaces (Dasch, 1982), flaming combustion in open furnaces (Gwaze et al., 2006) and incomplete combustion in boilers for wood, wood chips or pellets (Hindsgaul et al., 2000; Johansson et al., 2003; Wierzbicka et al., 2005). This suggests that carbon aggregates are emitted from a wider range of combustion conditions than the narrow temperature range around 1000 °C used in the present study. On the other hand, emissions from low-temperature combustion were found to be dominated by large spherical carbon particles (Paper I). Particles with similar morphology have previously also been observed from smouldering combustion, which is characterised by low combustion temperatures (Colbeck et al., 1997; Hallett et al., 1989; Pósfai et al., 2002). Thus, the morphology of wood combustion particles seems to vary with combustion conditions such as temperature and air supply. However, carbon aggregates and large spherical carbon particles also differ considerably with respect to structure (turbostratic vs. amorphous), chemistry (low vs. high content of organic carbon) and possibly solubility (insoluble vs. soluble) (Kjällstrand, 2002; Kjällstrand and Olsson, 2004; Pósfai et al., 2004), and these physicochemical properties may have a stronger influence on the pro-inflammatory potential of carbon aggregates and large spherical carbon particles than their morphology.

In the samples collected for biological experiments, the major morphological difference was the variation in the relative content of mineral particles and carbon aggregates (Paper II). The

two tunnel samples, that contained different amounts of these two particle classes, induced a similar pro-inflammatory response, indicating that these morphologies were not explanatory factors for the traffic-induced inflammation (Paper III). Furthermore, the model particles, containing mineral particles only (Porphyr, Quartz) or carbon aggregates only (Diesel), all induced a negligible pro-inflammatory response compared to the traffic-derived particles, and could therefore not explain the observed responses (Paper III and IV). In the literature, conflicting results are reported with respect to the relative potency of mineral particles and carbon aggregates (De Berardis et al., 2003; Hetland et al., 2000; Shao et al., 2006). A possible explanation is that the pro-inflammatory response is influenced by other particle characteristics, such as the structure of mineral particles (Øvrevik et al., 2005) or the content of organic compounds in carbon aggregates (Boland et al., 2000; Bonvallot et al., 2001).

Morphology has, to a certain extent, been found to influence the pro-inflammatory potential of differently shaped particles of the same material, e.g. hydroxyapatite (Grandjean-Laquerriere et al., 2005). However, for the particles from residential wood smoke and traffic included in the present study, particle morphology did not seem to be of primary importance for the pro-inflammatory response.

5.1.2 Primary particle diameter

The mean diameter of primary particles in carbon aggregates from wood smoke was significantly larger than for aggregates from traffic (Paper II). Based on Wittmaack's graphical presentation of the relationship between gas adsorption measurements and primary particle diameter measurements (Wittmaack, 2007), the surface areas for carbon aggregates from wood smoke and traffic were estimated to be 80 and 100 m²/g, respectively. Several studies have suggested that the surface area is the major explanatory factor for the inflammatory potential of low-solubility low-toxicity particles (Donaldson et al., 2008; Monteiller et al., 2007; Stoeger et al., 2006). However, in the present study, experiments with washed particles from wood smoke and diesel exhaust suggested that surface area did not influence the cytokine release from the monocytic cell line (Paper III). Possible explanations could be that some organic material still remained after organic extraction, or that the surface charge or metal content differed between the washed samples (Ghio et al., 2002; Veronesi et al., 2002b). Furthermore, aggregation properties could also affect the results, in causing differences in the particle surface area available for cellular contact. The primary particle

diameter would then be a poor measure for surface area. The present *in vitro* experiments with native particles were not suitable for investigations of the influence of surface area on the inflammatory response, since so many other physicochemical characteristics than the surface area differed between the samples, including the content of PAHs, organic carbon, mineral particles and endotoxin. In the experiments with native particles, the organic fraction accounted for up to 80 % of the cytokine release induced by wood smoke (Paper III). For the traffic derived particles, however, both the organic fraction and the content of endotoxin influenced the cytokine release, and accounted for almost 100 % of the response for Tunnel St-. It therefore seems evident that other particles characteristics than the particle surface area have a stronger influence on the pro-inflammatory potential of particles from wood smoke and traffic.

5.1.3 *Primary particle microstructure*

Combustion particles from traffic and high-temperature wood combustion were found to exhibit a similar graphitic character (Paper II). In contrast, particles from a range of residential wood stoves were recently reported to exhibit a less graphitic character than diesel exhaust particles in two studies using near-edge X-ray absorption fine structure spectroscopy (NEXAFS) (Braun, 2005; Braun et al., 2008). Whereas the wood smoke particles in the present study were collected by aerosol sampling (Paper II), Braun and co-authors analysed samples collected either from the interior walls of various wood stoves or from their chimneys. This difference in collection methods could lead to a selection of different populations of particles, which could explain the conflicting results concerning the similarity of the graphitic structure of wood smoke and diesel exhaust particles. Toxicological studies suggest that the crystal structure of ultrafine TiO₂ particles (anatase vs. rutile) influences the cytotoxicity and the release of the pro-inflammatory cytokine IL-8 induced by the particles (Sayes et al., 2006). Furthermore, Albrecht et al. (2007) reported that the cytotoxic and inflammatory effects of quartz particles were reduced by surface coating, suggesting that surface properties were important for the quartz-induced toxicity. If there is a source-dependent difference in the graphitic character, as reported by Braun and co-authors (2005, 2008), this could possibly influence the surface properties of particles from wood smoke and traffic and thereby their inflammatory potential.

The difference in turbostratic microstructure between primary carbon particles from wood smoke and traffic (one nucleus vs. several nuclei), probably reflected a difference in the particle formation process (condensation vs. coagulation/condensation) (Paper II). This difference in number of nuclei is not likely to affect biological responses, since cells interact primarily with the particle surface. Characterisation of carbon microstructure in ambient samples has, however, been suggested as a complementary tool in source apportionment studies, since the microstructure of primary carbon particles differs with combustion source (Hays and Vander Wal, 2007; Shulman S., 1997). Data concerning the graphitic character of carbon particles from residential wood combustion are conflicting (Paper II, (Braun, 2005; Braun et al., 2008)), and the characteristics of the microstructure of carbon aggregates emitted from different wood combustion conditions are not sufficiently described in the literature. Therefore, additional work remains before microstructure analyses can be successfully applied to complement source apportionment studies.

5.1.4 *Elemental composition*

In agreement with the findings of Hedberg et al. (2002) and Sällsten et al. (2006), carbon aggregates from wood smoke were characterised by K in the XRMA analysis (Paper I). Traffic-derived carbon aggregates were characterised by Si and Ca, and both these elements have previously been observed in carbon aggregates from diesel exhaust (BéruBé et al., 1999). For combustion particles, these three elements (K, Si and Ca) have not been associated with pro-inflammatory effects. Moreover, Ca and K have been suggested to be relatively inert and thus non-toxic (Pagan et al., 2003), as opposed to transition metals such as Zn, V, Cr, Ni and Cu that have been associated with inflammatory effects (Ghio et al., 1999; Kodavanti et al., 2008; Kodavanti et al., 1998; Schwarze et al., 2006). These transition metals may have been present in the combustion particles from wood smoke and traffic, but could not be distinguished from the background contributions from the microscope in the XRMA analysis (Section 4.2.2). Consequently, the pro-inflammatory responses to combustion particles from wood smoke and traffic observed in the present study could have been affected by transition metals.

5.1.5 Organic chemistry

The wood smoke particles from high-temperature combustion contained higher levels of organic carbon than the traffic-derived particles (Paper II). However, even higher levels of organic carbon have been reported for particles from residential wood combustion in the literature (Schauer, 2003; Tesfaigzi et al., 2002), probably due to application of less efficient combustion conditions with lower combustion temperatures. For the traffic-derived particles, comparison with the literature is difficult, since the total and thus the organic carbon content depends on the mineral particle content in the samples (Paper II, Table 2). The wood smoke samples contained from 25 to 150 times more PAHs than the traffic-derived samples (Paper II and IV). The PAH levels detected in the traffic-derived particles (73-381 ng/mg) were on similar levels as detected in diesel exhaust emissions (273-444 ng/mg) by Manoli et al. (2004). For wood smoke particles, the PAH levels have previously only been reported as ambient air concentrations (ng/m^3) or emission factors (ng/kg), but not as fractional mass abundances (ng/mg), and comparison with the literature is therefore not possible. The PAH content in wood smoke particles has, however, been reported to increase with the combustion temperature for incomplete combustion conditions (Kjällstrand and Olsson, 2004), suggesting that wood smoke particles from lower combustion temperatures may have a lower PAH content than the present wood smoke sample.

PAHs are the group of organic compounds that have been most extensively characterised in PM, both with respect to occurrence and biological effects (Marano et al., 2007). Linear regression analysis suggested that PAHs may influence the cytokine release induced by wood smoke and traffic-derived particles in the present *in vitro* experiments. However, comparison of the calculated PAH contents in the organic extracts and the biological effects induced by these extracts indicated that other organic compounds than the measured PAHs also influenced the responses (Paper III and IV). In the literature, both unsubstituted and substituted PAHs, such as nitro- and oxy-PAHs, have been suggested to be involved in the biological effects induced by ambient particles and extracts of wood smoke and diesel exhaust particles (Baulig et al., 2003; Bonvallot et al., 2001; Kubatova et al., 2006; Li et al., 2003b; McDonald et al., 2004a; Steerenberg et al., 2006; Xia et al., 2004). The organic fraction of particles from wood smoke and traffic comprises a large number of compounds besides PAHs, such as nitrated and oxygenated PAHs, aliphatic hydrocarbons, quinones, aldehydes, ketones, organic acids and various chlorinated organics (Hedberg et al., 2002; Marano et al.,

2007; Naeher et al., 2007; Zielinska et al., 2004). The biological effects of many of these compounds, and their contributions to particle-induced inflammation, are largely unknown. Even though substituted and unsubstituted PAHs are plausible candidates for the pro-inflammatory effects induced by the organic fraction of wood smoke and traffic-derived particles, other organic compounds could also have contributed to the pro-inflammatory responses.

The influence of different particle fractions on the pro-inflammatory response was investigated by comparison of the effects induced by native particles, organic extracts and washed particles (Paper III and IV). During exposure to wood smoke particles, the organic fraction was found to account for the majority of the pro-inflammatory response. Similarly, the biological effects of diesel exhaust particles have also been attributed to the organic fraction (Baulig et al., 2003; Bonvallot et al., 2001; Li et al., 2003a). For traffic-derived particles, however, the washed particles induced a greater cytokine release than the organic extract. The insoluble fraction of the washed traffic-derived particles comprised mineral particles from road abrasion and iron particles from brake wear, in addition to combustion particles. Washed combustion particles have generally been found to have a low pro-inflammatory potential (Baulig et al., 2003; Bonvallot et al., 2001; Li et al., 2003a), whereas mineral and brake wear particles have been associated with inflammatory responses (Becher et al., 2001; Gerlofs-Nijland et al., 2007; Øvrevik et al., 2005). Thus, the content of mineral and brake wear particles may partly explain the cytokine release induced by the washed traffic-derived particles. The contribution of endotoxin in the response to washed traffic-derived particles was not investigated, but particle-bound endotoxin could possibly also have influenced the observed cytokine release.

In the monocytic cell line, the much greater cytokine release induced by native particles as compared to the sum of the releases induced by the organic and washed particle fractions, suggested that the organic fraction must be adsorbed to the particles to exert biological activity (Paper III). Similarly, benzo(a)pyrene adsorbed to carbon black particles increased the release of TNF- α in a monocyte/macrophage cell line, whereas the two constituents did not have any effect separately (Chin et al., 1998). In the co-culture, however, the sum of the responses induced by the organic extracts and the washed particles was similar to the response to the native particles, suggesting that particle uptake was not required for activation of the

organic fraction in this system (Paper IV). This finding is supported by the majority of the studies comparing the pro-inflammatory response to native particles and organic extracts, which also report that these two fractions are equally potent (Baulig et al., 2003; Bonvallot et al., 2001; Rumelhard et al., 2007; Vogel et al., 2005). The literature and the present results from the two different model systems suggest that particle uptake is required for activation of the organic fraction in some model systems but not in others, but the involved mechanisms are unknown. In spite of these conflicting data, the overall response patterns were consistent in the two model systems, since the organic extract accounted for the majority of the response induced by the wood smoke particles, whereas the washed particle fraction was more potent for the traffic-derived particles in both model systems.

The reduction in the number of viable cells in the co-culture was associated with the organic fraction of particles from both sources, but was more pronounced for wood smoke than for traffic-derived particles (Paper IV). In accordance with this, the organic fraction of the wood smoke particles accounted for a larger part of the cytokine release than for the traffic-derived particles. The traffic derived particles induced a greater release of IL-6 and IL-8, with increasing exposure time, than wood smoke particles. This could be due to induction of a time dependent increase in cytokine release by washed particles but not by organic extracts. However, to determine the influence of the organic and washed particle fractions on the source-dependent difference in cytokine release, it is necessary to perform additional experiments investigating the time-course of the cytokine release induced by the organic and washed particle fractions.

5.1.6 Endotoxin

The endotoxin inhibitor polymyxin B sulphate reduced the cytokine release induced by the traffic-derived samples with 10 to 50 % (Paper III and IV). Since polymyxin only reduced the cytokine release partially, the data indicate that other particle characteristics also influenced the release of pro-inflammatory mediators, in accordance with previous data on ambient particles (Alfaro-Moreno et al., 2007a; Hetland et al., 2005). Furthermore, the cytokine release induced by the traffic-derived particles was significantly greater than that induced by wood smoke even in the presence of polymyxin, suggesting that the observed source-dependent difference in response patterns was not due to endotoxin contamination (Paper VI).

5.1.7 Implications of physicochemical characterisation

The electron microscopic analyses showed that carbon aggregates from wood smoke and traffic differed with respect to elemental composition, primary particle diameter and microstructure (Paper I and II). Lewis et al. (2003) used scanning electron microscopy in source apportionment to obtain additional information about sources with low contribution to the total mass of PM (Lewis et al., 2003). Moreover, Hays and Vander Wal (2007) recently suggested identification of carbon microstructure as a complement to existing source apportionment methods. Thus, a possible application of the source-specific differences in physicochemical characteristics identified presently (Paper I and II) could be to distinguish between carbon aggregates from residential wood combustion and traffic in future source apportionment studies.

The particle properties analysed by electron microscopic analyses (Paper II) could not be associated with the measured biological responses (Paper III). In contrast, two of the parameters determined by bulk chemical analyses (Paper II), the content of PAHs and organic carbon, were associated with the reduction in cytokine release induced by organic extraction (Paper III). The content of PAHs and organic carbon could, however, not be correlated with the cytokine release induced by the native particles. Therefore, a correlation analysis was not an appropriate strategy for the present samples in investigating the influence of particle characteristics on the inflammatory response. The reason may be that relatively few samples were included in each of the *in vitro* studies (Table 1), and that the variations in physicochemical characteristics between the samples were large. However, organic extraction and inhibition of endotoxin, did have an evident effect on the measured pro-inflammatory responses, and proved to be appropriate strategies (Paper III and IV). Overall, the present results demonstrate that it is necessary to combine several different approaches to identify the particle characteristics that influence the inflammatory potential of PM from different sources.

5.2 Pro-inflammatory effects of wood smoke and traffic derived particles

5.2.1 In vitro model systems

The pro-inflammatory potential of particles from wood smoke and traffic was investigated in two *in vitro* model systems in two separate studies (Paper III and IV). The experimental setup in Paper IV was designed to do a more thorough investigation of the pro-inflammatory effects

of PM from the two sources rather than for comparison of the responses in the two model systems. Therefore, the cell numbers and the volume per well differed between the two model systems, as well as the applied particle concentrations. A direct comparison between cytokine levels released in the two model systems is, therefore, not possible. In order to investigate the influence of cell to cell interactions on the release of pro-inflammatory mediators, it would have been necessary to compare the responses in both the co-culture and mono-cultures of both cell types, cultured with the same cell numbers and medium volumes per well.

Although the two studies were not designed for comparison, they had some common features. The time point of 12 h was applied in both model systems, as were two of the particle samples, Tunnel St- and Wood. For the comparable time point and particle samples, the pro-inflammatory response was found to correspond very well. There was also a good agreement with respect to the role of IL-1 in the early pro-inflammatory response, which seemed to be more pronounced for traffic-derived particles than wood smoke. Furthermore, the *in vitro* results from organic extraction and inhibition of endotoxin corresponded well between the two studies (Paper III and IV). The only discrepancy between the data from the two model systems was that the sum of the responses induced by the organic fraction and the washed particles was much lower than the response induced by the native particles in the monoculture (Paper III), but not in the co-culture (Paper IV), as discussed in section 6.1.5.

5.2.2 Differential pro-inflammatory response patterns

In the co-culture of monocytic and pneumocytic cell lines, wood smoke particles induced a lower level of IL-6 and IL-8 release than the traffic-derived particles after 40-64 hours exposure (Paper IV). Wood smoke particles from different combustion conditions also induced a lower cytokine release, confirming that this low response was not specific for particles from high-temperature combustion (Schwarze et al., 2008). IL-6 activates the immune system through activation of B-lymphocytes and monocytes and contributes to systemic inflammation by induction of acute-phase protein synthesis (Mills et al., 1999), while IL-8 contributes to pulmonary inflammation by attracting and activating neutrophils (Dinarello, 2000). Thus, the induction of greater levels of IL-6 and IL-8 by traffic-derived particles, may suggest that traffic-derived particles induce a more severe effect than wood smoke particles on both pulmonary and systemic inflammation.

Exposure to Wood, and to the wood smoke samples from different combustion conditions, reduced the number of viable cells compared to the unexposed controls, while the traffic-derived particles induced a less pronounced effect (Paper IV, (Schwarze et al., 2008)). All the particle samples induced very low cytotoxicity, and the necrosis measured as LDH release was too low to explain the reduction in cell numbers. Similarly, the percentage of apoptotic cells did not change during exposure, suggesting that the reduced cell numbers were not caused by apoptosis. These data indicate that particle exposure induced a reduction in proliferation. In support of this, a particle-induced reduction in proliferation has previously been observed in both monocyte/macrophage and epithelial cell lines (Möller et al., 2002; Poma et al., 2006; Zhang et al., 2007). Reduced proliferation has also been observed in the proximal alveolar region of the lung in neonatal rats after particle exposure (Pinkerton et al., 2004). A particle-induced reduction in proliferation could be due to cell cycle arrest (Deng et al., 2007; Zhang et al., 2007), induced by for instance DNA damage (Okada and Mak, 2004). Interestingly, in a separate study, Wood was found to induce DNA damage to a greater extent than Road tunnel in monocultures of the monocytic and epithelial cell lines used in Paper IV (Danielsen et al., 2008). A possible explanation is, therefore, that DNA damage influenced the reduced cell numbers observed in the co-culture during exposure to wood smoke.

A consequence of a particle-induced reduction in proliferation, in general, could be altered growth in the gas exchange region, that may be associated with impaired development of lung structure and function in young children. Epidemiological studies have associated exposure to ambient air pollution, including PM, with reduced lung function development (Gauderman et al., 2004; Gauderman et al., 2007; Jedrychowski et al., 1999). Furthermore, exposure to increasing levels of PM₁₀ has, for healthy children, been associated with increased carbon content in airway macrophages and decreased lung function (Kulkarni et al., 2006).

If the alveolar lung epithelium is damaged, the normal repair process occurs through proliferation of type II epithelial cells. Therefore, a reduced proliferation of lung epithelial cells, as observed in an epithelial cell line in this study, might also lead to inadequate repair of tissue damage, which could in turn lead to bronchiolization or fibrosis (Geiser, 2003a; Jensen-Taubman et al., 1998; Serrano-Mollar et al., 2007). IL-1 β is an early pro-inflammatory cytokine that can induce release of a range of pro-inflammatory cytokines, but it has also been found to enhance alveolar epithelial repair (Chung, 2001; Dinarello, 2000; Geiser et al.,

2000; Geiser, 2003b). In the *in vitro* systems used presently, IL-1 seemed to play a more important role in the pro-inflammatory response induced by traffic-derived particles as compared to wood smoke particles (Paper III and IV). Since inhibition of IL-1 β has indicated that this cytokine is necessary for alveolar epithelial repair *in vitro* (Geiser, 2003b), a lack of IL-1 activation during exposure to wood smoke particles could possibly affect tissue repair, although other mediators such as the keratinocyte, hepatocyte and fibroblast growth factors also contribute to the alveolar repair process (Geiser, 2003a; Warburton and Bellusci, 2004; Ware and Matthay, 2002).

In vitro studies with cell lines are widely used and easy to perform, but have several drawbacks such as altered characteristics and limited communication with other cells compared to cells in tissue. Furthermore, *in vitro* model systems only provide information about signalling molecules and viability which can not predict the more complex biological responses, such as cellular influx and tissue damage, in humans. Thus, the interpretations of the present *in vitro* results with respect to possible effects on pulmonary inflammation and repair need to be confirmed in *in vivo* model systems.

5.2.3 Source comparison

In the *in vitro* experiments, PM from the two sources was compared on an equal mass basis. This approach is supported by some studies reporting that particles from wood smoke and traffic may be present in similar concentrations in ambient environments (Glasius et al., 2006; Saarikoski et al., 2008; Song et al., 2007; Wu et al., 2007). The literature does, however, give insufficient information with regard to the exposure to particles from residential wood combustion, both with respect to the concentrations and the physicochemical characteristics of wood smoke particles in ambient air. Moreover, since the pulmonary deposition and retention of particles partly depends on the physicochemical particle characteristics (Kreyling et al., 2007; Löndahl et al., 2007), the pulmonary cells may still be exposed to different concentrations of particles from wood smoke and traffic, even during exposure to equal concentrations of particles from the two sources.

The applied wood smoke particles were collected during high-temperature combustion only, but as described above, the cellular responses were very similar for PM collected during more variable combustion conditions. This suggests that the present results reflect the pro-

inflammatory potential of combustion particles from a range of combustion conditions. However, the samples were collected in a laboratory, and the organic fraction of wood smoke particles has been suggested to be transformed by photochemical reactions in ambient air to a higher extent than traffic-derived particles (McDow et al., 1994; Vione et al., 2006). Furthermore, ambient chemical transformation of the organic fraction has been suggested to reduce the pro-inflammatory effects of ambient particles (Jalava et al., 2006). The samples included in the present study were, however, not suitable for investigations of the influence of ambient chemical transformation on the pro-inflammatory responses. The traffic derived samples were considered to be relevant for the particles inhaled during ambient traffic exposure. However, PM collected by filtration or impaction may have altered physicochemical characteristics compared to ambient PM, both due to transformation processes on the collection substrate and due to the vigorous methods necessary to extract the particles from the substrate (Ayres et al., 2008). Due to the disadvantages inherent in the applied sampling procedures and the limited selection of particle samples investigated, additional experiments are needed to verify the relevance of the present results.

6 Conclusions

In this study we have characterised the physicochemical properties of particles from residential wood smoke and traffic and compared the pro-inflammatory potential of PM from these two sources in *in vitro* model systems. We also investigated the influence of physicochemical particle characteristics on the pro-inflammatory response. Particles from the two sources were found to induce similar responses after 12 hours exposure, but during increasing exposure time, up to 64 hours, traffic-derived particles induced a greater release of pro-inflammatory cytokines and a less pronounced reduction in the number of viable cells. Thus, the results indicate that particles from different sources may affect different biological parameters. A possible consequence of the observed source-dependent difference in the pro-inflammatory response patterns is that particles from the two sources induce a differential immune response after deposition in the lung. A greater release of pro-inflammatory cytokines, induced by traffic-derived particles, may result in a more pronounced influx of inflammatory cells to the lung or a more severe systemic inflammation. On the other hand, the greater reduction in cell number caused by wood smoke particles could possibly impair tissue repair processes in the lung or lung development. However, *in vivo* studies are necessary to gain more detailed knowledge on how particles from wood smoke and traffic may affect the immune response differentially.

The physicochemical characteristics of particles from wood smoke and traffic differed with respect to elemental composition, primary particle diameter, microstructure and content of PAHs and organic carbon. In the present model systems, the pro-inflammatory response was, however, only influenced by the content of organic carbon and the measured PAHs. In addition, organic extraction and inhibition of endotoxin induced significant decreases in the pro-inflammatory response. Although the electron microscopic analyses provided detailed information about the physicochemical characteristics of carbon aggregates, it was not possible to link these characteristics to the observed pro-inflammatory responses. Thus, the results demonstrate that it is necessary to combine several different approaches to identify the particle characteristics that influence the pro-inflammatory potential of PM. The source-specific differences for combustion particles from wood smoke and traffic detected by electron microscopic analyses could, however, be used to complement existing source apportionment methods.

The influence of different particle fractions on the pro-inflammatory response was found to depend on the particle source. The organic fraction accounted for the majority of the cytokine release induced by wood smoke particles, whereas for the traffic-derived particles the washed fraction induced the greatest release. The reduced cell numbers were on the contrary associated with the organic fraction for particles from both sources. Overall, the organic fraction was the particle fraction that exerted the strongest influence on the measured biological endpoints, and a reduction of the organic fraction of PM emissions could possibly be a useful approach for environmental strategies to reduce emissions of hazardous PM components. Reduction of the organic fraction may be achieved in vehicles by improvement of exhaust after-treatments such as catalysts (Boland et al., 1999; McDonald et al., 2004b) or in residential wood combustion by replacing conventional wood stoves with new-technology stoves that provide more complete combustion conditions (Boman et al., 2003b; Johansson et al., 2004; Kjällstrand and Olsson, 2004).

Although the results from *in vitro* model systems can not be extrapolated to humans, the present findings lend support to epidemiological and human inhalation studies which suggest that wood smoke particles may induce adverse health effects. In the *in vitro* experiments, particles from residential wood combustion and traffic both induced significant effects, but different parameters were affected. This suggests that wood smoke particles may induce different biological effects than traffic-derived particles, but be equally harmful. Further studies are, however, needed to characterise and compare the biological effects of particles from wood smoke and traffic.

7 Future perspectives

In the present *in vitro* studies, the organic fraction was identified as the most potent fraction of the PM, but the individual organic compounds or groups of compounds that were involved in the pro-inflammatory responses were not investigated. Application of fractionation of organic extracts in combination with chemical analysis has been successfully applied by Xia et al. (2004) and Kubatova et al. (2006) to determine the groups of organic compounds involved in inducing cellular oxidative stress. Similar investigations should be performed for a wider range of particle samples and biological endpoints. From a toxicological point of view, it is important to identify the fractions of PM that induce a response, and target the chemical analyses to these fractions. In contrast, the aerosol scientists, that usually perform the chemical analyses, focus on tracers that are specific for a certain source, but may not affect particle toxicity. One example of such a 'biologically inactive tracer' is potassium, which has frequently been used to trace wood smoke emissions. In order to identify physicochemical characteristics that influence the biological effects of PM, a stronger collaboration between aerosol scientists and toxicologists would be advantageous.

Future experimental toxicology studies should apply samples that are relevant for human exposure, both with respect to collection methods and sampling sites. Sampling of PM into liquid has been demonstrated to not introduce alterations with respect to physicochemical particles properties (Ayres et al., 2008; Kim et al., 2001), and might be an appropriate collection method for *in vitro* experiments. With respect to sample selection, ambient wood smoke particles have not yet been applied in experimental toxicology. Furthermore, the influence of differences in wood-combustion conditions on the particle toxicity is unknown. In order to target the strategies applied to reduce wood smoke emissions in the northern countries, biological experiments are needed to determine how the combustion conditions influence the particle toxicity, and to determine the toxicity of the emissions from new stoves with improved combustion technology.

The present *in vitro* results should be verified by performing inhalation studies in animal models and humans. PM exposure has been associated with increased morbidity and mortality, particularly for susceptible individuals. Since relatively mild effects were observed in healthy rats after exposure to wood smoke and diesel exhaust particles (Seagrave et al., 2005), it would be interesting to apply a susceptible animal model to compare the negative

effects induced by particles from the two sources. One approach would be to use models for cardiovascular disease, such as apolipoprotein E-deficient mice or spontaneously hypersensitive rats (Araujo et al., 2008; Cao et al., 2007; Corey et al., 2006; Gilmour et al., 2004; Wallenborn et al., 2007). The published human inhalation studies have not been designed to compare the effects induced by different sources (Barregard et al., 2006; Barregard et al., 2008; Larsson et al., 2007; Stenfors et al., 2004). However, comparative studies would be a useful tool in the process of targeting strategies for reducing human PM exposure to the appropriate particle sources.

8 References

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